

A STUDY ON THE ROLE OF A CLOSE HOMOLOGUE OF *Bacillus cereus* ISOLATED FROM *Metaphire posthuma* ON GERMINATION OF GRAM (*Cicer arietinum*) SEEDS FOR ITS USE AS BIOFERTILIZER

Sreejata Biswas¹, Pulak Lahiri² and Satadal Das³

¹ Department of Zoology, Bangabasi Morning College,
19, Rajkumar Chakrabarty Sarani, Kolkata 700009

² Formerly of Department of Zoology,
University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019

³ Peerless Hospital and B.K. Roy Research Centre,
360 Panchasayar, Kolkata 700094.

Abstract

Earthworms have intimate association with soil microorganisms which are often symbiotic and resident in their gut. The present study isolated the most predominant bacterium from *Metaphire posthuma*, commonly occurring earthworm in Indian soil. The microbe was identified as *Bacillus* sp, a close homologue of *Bacillus cereus*. Gram seeds were allowed to germinate at room temperature in the laboratory in presence of the *Bacillus* isolate in different media. Germination rates increased by 2- 8.3 % and shoot length and root length of germinated seeds increased significantly indicating the bio-fertilizer role of this microbe.

Key words: *Earthworm. Metaphire posthuma, Bacillus sp, germination, biofertilizer.*

INTRODUCTION

The success of a flowering plant depends on efficient seed germination. Among many animals that help in plant propagation earthworms are a dominant group. Anecic earthworms are important dispersers and predators of plant seeds. Generally, seed burial by anecic earthworms is thought to be primarily beneficial, by reducing seed predation on the soil surface and, in addition, by creating gaps for seed germination and nutrient- rich regeneration niches in earthworm middens [1]. Beside the direct effects by ingestion of plant seeds, earthworms have other influences on germination of seeds. Earthworm casts besides being a good soil fertility enhancer have beneficial role in seed germination too. Hidalgo *et al* (2005) in their experiment with cucumber (*Cucumis sativus*) seeds found that rate of germination and seedling growth increased in presence of earthworm (*Eisenia foetida andrei*) castings [2]. Arancon *et al* (2012) found that soaking seeds in vermicompost extracts significantly increased germination percentage and seedling growth of tomato and lettuce compared with control [3]. In a study Lazcano *et al* (2010) observed that incorporation of vermicompost in the growing media of maritime pine (*Pinus pinaster* Ait.) increased germination by 16% [4]. Beside increased nutrient availability in presence of earthworm excreta, earthworm casts were shown to accelerate seed germination by increasing water permeability of the seed surface [5] and by breaking seed dormancy [6]. Moreover, earthworm excreta were shown to contain rhizogenic substances similar to indole acetic acid [7] and are able to alter protein synthesis in seedlings [8].

Earthworm casts contain greater number of microbes than the soil they live in [9, 10, 11,12]. Experiments on several tropical earthworm species have shown that the enzymes like cellulase and mannase found in the gut content are produced by ingested bacteria and not by cells of the gut wall [13, 14, 15]. Thus earthworms provide suitable site in their gut for the vigorous increase in microbial number that help in the decomposition process. These microbes in soil remain in a dormant state and can become active only in the suitable environment [16]. Soil enrichment and facilitation of plant growth are not done by the enzyme mediated decomposition process only, but also with the help of some growth regulators and vitamins secreted by the microbes. Atlaviniyte and Daciulyte (1969) observed that *Allolobophora rosea*, *A. caliginosa*, *L. terrestris* and *L. rubellus* increased the microbial population in soil upto 2-3 folds than the control soil, which in turn helped to increase the amount of vitamin B₁₂ upto 2-7 folds [17].

Micro organisms have effective role in seed germination. Higa (1991) found that on treating the seeds with effective microorganisms, EM, a microbial inoculant comprised mainly of lactic acid bacteria, photosynthetic bacteria, yeasts and actinomycetes that are commonly found in soil, increased the rate of germination [18]. In an experiment to investigate the role of bacteria *Pseudomonas* sp, *Klebsiella oxytoca* and *Enterobacter sakazakii* in the germination of *Striga hermonthica*, Babalola *et al*, (2007), found that bacterial isolates could stimulate *S. hermonthica* germination in the laboratory [19]. Seed germination of alfalfa improved and seedlings with larger roots were found when the seeds were inoculated with plant growth promoting bacterial strains grown in iron deficient minimal medium than the uninoculated control seeds [20]. In an experiment Koh and Song (2007), isolated purple nonsulfur bacteria *Rhodospseudomonas* sp strain KL9 and found it to produce indole-3-acetic acid (IAA) and 5-aminolevulinic acid (ALA) that helped in increase in germination percentage of tomato seed, total length and dry mass of germinated tomato seedling by 30.2%, 71.1% and 270.8% respectively compared with those of the un- inoculated control [21].

In view of the above the present study was undertaken to investigate the role of selected earthworm gut bacteria in seed germination and growth of seedling with an objective to use the bacteria as an organic agent in green agriculture.

MATERIALS AND METHODS

Materials

Metaphire posthuma, a geophagous earthworm was selected which is commonly found in various parts of India, specially in the gangetic plains [22]. Gram seeds (*Cicer arietinum*, product no. Mahamaya IIB115) were used for studying germination.

Methods

Isolation of gut bacteria from earthworms

Earthworms were pinned on dissecting tray and the dorsal surface was wiped with cotton moist in alcohol. The gut wall was opened by using aseptic instruments. A sterile bacteriological loop was used to collect gut content from the hindgut and streaked on plates of nutrient agar and Mac Conkey Medium.

Observation of Bacterial Colonies

Bacterial colonies with different morphological appearances were observed on bacteriological media and the colonies were counted. The colony count technique had been routinely used. The mean number of colony forming units (cfu) of bacteria, collected from forty earthworm samples was calculated. The most predominant bacterium was named as *Bacillus* sp # 203. Morphologically the colonies had licheniform shape, moderate size, flat elevation, irregular margin and pink colour in Mac Conkey Medium. The microbe in question, Gram positive in nature, was selected for phylogenetic analysis by 16S rRNA molecular technique.

Genetic Identification of predominant gut bacterium isolated

Genomic DNA was isolated from the pure culture at the laboratory of GeNei™, Bangalore, India. Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using Taq DNA Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe from National Center for Biotechnology Information (NCBI GenBank) and The Ribosomal Database Project (RDP database).

Germination of Gram seeds:

Bacillus sp. # 203 after separation was suspended in sterile distilled water according to Mac Farland's standard solution so that 1ml of the suspension contains 10^8 bacteria. It was allowed to incubate for 4 hours at 37°C. Seeds were treated with antifungal agent prior to the experiment. The seeds were allowed to germinate at room temperature in the laboratory according to inclined glass plate-blotter method [23].

Each sterile glass plate contained nine seeds. Six such plates were kept as control in sterile distilled water. Another set of six plates containing also nine seeds each, was used as test, to which the bacterial suspension was applied. For each plate, 9 ml of sterilised water or bacterial suspension was added. After 7 days, the root and shoot lengths of the seedlings were measured separately and the rate of germination was calculated.

The process was tested in various medium for growth of the bacteria, viz. peptone, glycine, in a mixture of soil and water and only water.

RESULTS

Genetic identification

Based on nucleotides homology and phylogenetic analysis, the microbe was detected to be *Bacillus* sp. (GeneBank Accession Number: EU236738). Nearest homolog species was found *Bacillus cereus* (Accession No. EU871042).

Results of seed germination

Effects of germination of gram seeds by application of *Bacillus* sp#203 in different media are tabulated in Table No.1 to 6.

Table No. 1: Germination of gram seeds in presence of *Bacillus* sp. # 203 in glycine (concn.M/20)

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	5.81 \pm 3.56	5.61 \pm 3.44
Root	5.65 \pm 3.49	6.15 \pm 3.65*
%of germination	88.89	96.3
Increase in Germination percentage		8.3 %

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0. 5 to 0.4 levels
(t value \pm 0.72)

Table No. 2: Germination of gram seeds in presence of *Bacillus* sp. # 203 in peptone (undiluted)

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	3.39 \pm 1.52	3.76 \pm 2.08*
Root	3.67 \pm 1.29	3.73 \pm 1.27
%of germination	96.3	96.3

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0. 2 to 0.1 level
(t value \pm 1.05)

Table No. 3: Germination of gram seeds in presence of *Bacillus* sp. # 203 in peptone (10X dilution)

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	4.08 \pm 2.29	4.84 \pm 2.21*
Root	12.28 \pm 5.37	12.88 \pm 4.99
%of germination	88.89	90.74
Increase in Germination percentage		2.1%

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0. 1 to 0.05 level
(t value \pm 1.74)

Table No. 4: Germination of gram seeds in presence of *Bacillus* sp. # 203 in peptone (100X dilution)

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	3.35 \pm 2.42	3.39 \pm 2.28
Root	11.38 \pm 6.55	12.45 \pm 6.17*
%of germination	81.48	87.04
Increase in Germination percentage		6.8%

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0. 4 to 0.2 levels
(t value \pm 0.87)

Table No. 5: Germination of gram seeds in presence of *Bacillus* sp. # 203 in water and soil

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	4.89 \pm 2.298	5.12 \pm 2.17*
Root	13.36 \pm 6.70	12.86 \pm 5.86
%of germination	90.74	92.6
Increase in Germination percentage		2%

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0. 5 level
(t value \pm 0.53)

Table No. 6: Germination of gram seeds in presence of *Bacillus* sp. # 203 in water

Part of the seedlings	Control(Autoclaved distilled water) Mean length(cm) \pm SD	Test (Bacterial suspension) Mean length(cm) \pm SD
Shoot	4.40 \pm 1.98	5.11 \pm 1.91*
Root	10.99 \pm 5.08	11.82 \pm 5.29
%of germination	90.74	92.6
Increase in Germination percentage		2 %

SD= Standard Deviation

* 'p' value of difference between test and control is significant at 0.05 level
(t value \pm 1.87)

DISCUSSION

In an experiment Owa *et al* (2008) have observed that the earthworm products leave enough residues in the soil to affect post-drying germination to such an extent that even after five months of dryness and in complete absence of earthworms in soil the castings improve total germination [24]. Ayanlaja *et al* (2001) found that earthworm products i.e. earthworm casts in facilitating germination preferably affect radicle growth and elongation [6]. Thus, before depletion of the endosperm the embryo has successfully germinated, the root has begun to draw from extra- endospermic resources of the soil, and the seedling is ready for autotrophic photosynthesis activities.

Sharma *et al* (2007) found that phosphate solubilizing bacteria *Pseudomonas fluorescens* and *Bacillus megaterium* isolated from soil sample could enhance seedling length of *Cicer arietinum* [25]. They concluded that the use of these bacteria in the form of biofertilizer should be promoted. The present

work reveals that *Bacillus* sp # 203, isolated from common Indian earthworm *M. posthuma* also possesses such important role since germination rates as well as shoot length and root length of germinated gram seeds increase in all these instances. In another experiment the authors observed that gut bacterium of *M. posthuma* also facilitate paddy seed germination [26]. Inoculation of *Bacillus licheniformis* NCCP-59 improved seed germination of two rice varieties (Basmati-385 (B-385) and KSK-282) under Nickel stress [27]. Agrawal and Agrawal (2013) found five isolates of *Bacillus* sp from different rhizospheric soil of tomato crop in the vicinity of Dehradun to have the ability to produce IAA and all these strains, HBSVIII, FAR-IIIb, HBR-II, GAR-III and HBR-VII, significantly improved seed germination of tomato when compared to the uninoculated control [28].

In an experiment basal medium supplemented with 2g/l peptone was found to be effective for enhancing germination of orchid *Epidendrum ibaguense* [29]. In the present study bacterial suspension when made in peptone medium it was found that the percentage of seed germination was highest i.e. 96.3% in undiluted peptone medium. However the seedling growth was effectively observed in a ten times dilution of the medium. When the bacterial suspension was prepared in glycine the rate of germination increased without much significant increase in the seedling growth. However, in the later experiments the bacterial suspension was made in either water or in a mixture of soil and water and not in any other medium for bacterial growth because in a medium that contains ingredients which may have direct effect on seed germination may affect the result, for example, amino acids, amides and vitamin content of peptone are suspected to be responsible for enhancing seed germination [30]. At the same time the effective cost of production of the biofertilizer may be minimized by making the bacterial suspension in water.

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